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Concerning the wound-healing properties of *Sphagnum* holocellulose: the Maillard reaction in pharmacology

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Abstract

Sphagnum wound dressings can be 3–4 times as aborbent as cotton equivalents, but they also react chemically with proteins of all kinds. This reactivity gives them the potential of immobilizing whole bacterial cells as well as the enzymes, exotoxins, and lysins secreted by the most invasive pathogens. Once immobilized, enzymes and (by inference) exotoxins and lysins are rapidly inactivated by a Maillard reaction. The complex pectin in *Sphagnum* is structurally similar to known, immunostimulatory pectins from other plants, including some that are traditionally used for wound healing.

Keywords: *Sphagnum* mosses; Wound healing; Non-cytotoxic bacteriostasis; The Maillard reaction

1. Introduction

Sphagnum mosses have been used to dress wounds since the Bronze Age, and during the 1914–1918 war they were used extensively as field dressings because of their apparent efficacy in reducing the incidence of gas gangrene (Williams, 1982; Varley and Barnett, 1987a). Controlled, histologically monitored trials have confirmed that such dressings promote the healing of clean wounds, surgically created in pigs (Varley and Barnett, 1987b). The experimental infection of such wounds is, however, prohibited by ethical constraints and reports of a specific, antimicrobial or antivirulent effect, though numerous and mutually supportive, are still essentially anecdotal. Patents describing the hydroponic cultivation of suitable *Sphagnum* species under sterile conditions (Carus and Scales, 1985) and the incorporation of the leaves into composite wound dressings (Carus and Scales, 1986/1987) have been published.

In the absence of other evidence, the wound-healing effect was attributed exclusively to the mosses' ability to absorb and immobilize 20–25 times their own weight of fluid. This interpretation, and perhaps also the cost of cultivating *Sphagnum* under sterile conditions, seems to have redirected attention from *Sphagnum* to hydrocolloids such as alginates (Thomas, 2000). Fabrics prepared from calcium alginate or mixed calcium/sodium alginate fibres can rival *Sphagnum* leaves for absorbtivity, while alginates are freely available on the commercial market, and are easier to purify and sterilize. The outcome was that plans to manufacture the *Sphagnum*-based dressings on a commercial scale were set aside and the patents were allowed to lapse (Barnett, Carus and Scales, personal communications).

Meanwhile, discoveries were being made which suggested that the wound healing may be promoted, not by absorbtivity alone, but also by the chemical reactivity of something in the moss. A pectin-like polysaccharide ('sphagnan') containing highly reactive α-keto-carboxylic acid (–CO–CO_2H) groups had just been identified as a major component of the hyaline cell walls of *Sphagnum* mosses (Painter, 1983) when, in August 1984, a well-preserved human body, 2000 years old, was discovered in a *Sphagnum*-dominated peat bog ('Lindow Moss') in northern Cheshire, UK (Stead et al., 1986). An investigation sponsored by the British Museum soon established that the skin and other collagenous tissues of the body were tanned like leather and had acquired the colour of black coffee (Stead et al., 1986). These were obviously chemical reactions, and it was later found that the tanning agent was sphagnan (Painter, 1991a,b, 1995). Its keto-groups had condensed with pendant amino- and amido-groups in the collagen fibres, thereby cross-linking them. The resultant macromolecular complex had then undergone a Maillard reaction with production of the dark-brown pigment ('melanoidin') that is a characteristic end-product of this

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reaction (Painter, 1998). The reaction is easily reproducible in the laboratory with pigskin or salmon skin, and it has also been shown to preserve whole fish (Børsheim et al., 2001a).

In freshly-harvested *Sphagnum* and in the pure-white holocellulose that can be prepared from it by chlorite bleaching, sphagnan is covalently linked to cellulose and other polysaccharides in an insoluble complex (Painter, 1983, 1991b). In this form it cannot tan skins immediately, though over time it is gradually liberated by autohydrolysis in a soluble form that can do this (Painter, 1991b). The pores of the empty, hyaline cells of the mosses are, however, about 13 μm in diameter, allowing easy access to bacteria and virus particles. Proteins that are insoluble in the microscopic sense, such as the flagella or somatic proteins of bacteria or the proteinaceous coats of viruses, could therefore react relatively rapidly with the carbonyl groups in the insoluble, cell-wall matrix. Soluble proteins and polypeptides, including tissue-digesting exo-enzymes such as bacterial collagenases and hyaluronidases, and exotoxins and lysins of all kinds, should, however, react the fastest and become immobilized within the fragments of insoluble leaf material. Some simple experiments are now described which help to confirm these expectations and also provide a clue as to the fate of the immobilized biomolecules.

2. Methods and results

2.1. Immobilization of whole bacterial cells

Portions of *Sphagnum palustre* L. (Sphagnaceae) holocellulose were enclosed in sachets sewn from plankton netting of pore size 20 μm. The sachets were suspended separately in physiological saline in Erlenmeyer flasks, which were then autoclaved. The flasks were inoculated with varying proportions of liquid cultures of bacterial cells in their stationary phase of growth, and transferred to a mechanical shaker at 20 °C. At intervals, samples of the ambient cell suspensions were withdrawn for plate counts, which showed that the densities of bacterial cells had decreased to constant values after 20 h. The results obtained with the unmodified holocellulose are shown in Table 1. The controls were pure wood cellulose and *Sphagnum* holocellulose that had been treated with aqueous sodium borohydride to reduce its carbonyl groups (Børsheim et al., 2001a); neither showed significant immobilization of bacterial cells.

Table 1
Immobilization of whole bacterial cells on *Sphagnum* holocellulose

Culture	Percent remaining in suspension
Pseudomonas sp.	<0.4
Escherichia coli	<0.25
Bacillus sp.	<10
Micrococcus sp.	<0.1

Table 2
Firmly-bound protein [6.25 × N (%)] in *Sphagnum* holocellulose after treatment with different, soluble proteins and enzymes

Material	Source	Protein in complex (%)
Pepsin	Gastric juice	1.8
α-Amylase	*Aspergillus oryzae*	2.6
α-Amylase	*Bacillus licheniformis*	3.0
Invertase	*Candida utilis*	4.4
Serum albumin	Bovine	6.1
Hemoglobin	Human	12.3
Protease	*Streptomyces griseus*	13.3
Trypsin	Hog pancreas	27.6

Unbleached moss and peat gave results similar to those in Table 1 (Børsheim et al., 2001b).

2.2. Immobilization of soluble proteins and enzymes

The test material (50 mg) in water (2 ml) was sorbed on to *Sphagnum* holocellulose (Na^+ salt, 100 mg) and the moist particles were freeze-dried. After keeping for 48 h over anhydrous silica gel *in vacuo* at 4 °C the dry solid was washed exhaustively with ice-cold 0.5 M sodium chloride, followed by much distilled water. It was then freeze-dried again, and analyzed for nitrogen (Table 2). The proportion of firmly-bound protein is strongly correlated with the protein's basicity, but even the most acidic protein (pepsin) is bound to a significant extent.

2.3. Spontaneous inactivation of immobilized enzymes

The Maillard reaction brings about radical changes in both the covalent chemical structure and the conformation of any protein, and there seems to be no exception to the rule that enzymes and other biologically active proteins lose their activities in this process (Painter, 1998). The Maillard reaction that occurs with sphagnan is, however, different from that which occurs with simple sugars in one important respect; it is much faster, and will occur very rapidly at body temperature.

Particles of holocellulose carrying immobilized enzymes were weighed out and assayed for hydrolase activity by standard procedures (Dawson et al., 1986). In most cases, no residual activity was found. Immobilized invertase, after storage for 48 h at 4 °C, showed only ∼10% of the activity expected from the nitrogen content (Table 2) of its holocellulose complex; at 55 °C and pH 4.5 the residual activity decayed with a half-life of ∼3 h. The longest half-life (∼30 h at 20 °C and pH 6.9) was found for the exceptionally thermostable α-amylase of *Bacillus licheniformis* (Fig. 1). This was slow enough to permit quantitative monitoring of its denaturation as follows.

A glass column (100 mm × 9 mm) was packed with *Sphagnum palustre* holocellulose (500 mg) containing immobilized α-amylase (3% w/w) and irrigated at 25 °C and 0.3 ml min^{-1} by soluble starch (0.1% w/v) in 20 mM sodium

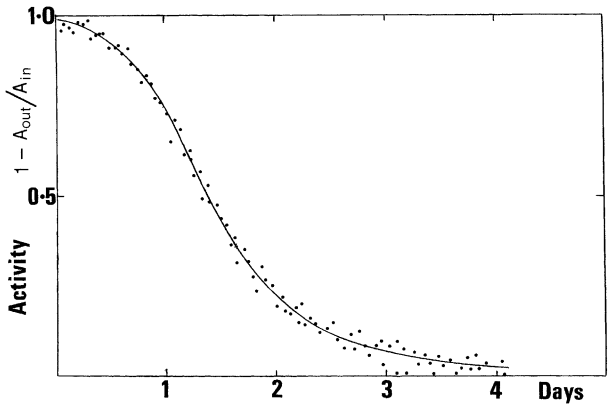

Fig. 1. Spontaneous decay in the activity of the α-amylase of *Bacillus licheniformis* immobilized on *Sphagnum palustre* holocellulose.

phosphate buffer (pH 6.9) containing 5 mM sodium chloride. Fractions (18 ml) were collected, mixed with aqueous iodine (1.6 mg I_2 in 2 ml 50 mM KI) and their absorbances at 550 nm were measured. The degree of amylolysis (ordinates, Fig. 1) was expressed as $[1 - A_{out}/A_{in}]$. A simple solution of the enzyme in the aqueous buffer lost no activity over the same period (7 days) at the same temperature. The N content of the holocellulose complex (Table 2) was the same after the experiment as before.

2.4. Sphagnan is not cytotoxic; it works by nutrient deprivation

To demonstrate this, sphagnan was prepared in soluble form (Painter, 1991b; Børsheim et al., 2001a). In Rideal–Walker tests with *Pseudomonas aeruginosa*, its 'phenol coefficient' was zero. In media containing nitrate as the only nitrogen source, the growth rate was unaffected even by high concentrations (>25 mg ml^{-1}) of sphagnan, whereas with ammonia as a nitrogen source, there was a threshold concentration of sphagnan above which growth was strongly inhibited.

This lack of cytotoxicity on the part of sphagnan, and its ability to inhibit growth only by sequestering ammonia and other primary amines in the ambient medium, must reflect its polymeric and hydrophilic character, which would oppose its traversing the cell wall and cytoplasmic membrane of bacteria. Similar arguments apply to the plasma membrane of mammalian cells, and the intense proliferation of leucocytes at the interface between wounds and *Sphagnum* dressings (Fig. 2) seems to confirm that the latter are not cytotoxic.

3. Discussion

Although they are of a preliminary nature, these simple experiments clearly demonstrate that *Sphagnum* wound dressings are much more than inert, absorbent materials, and their traditional use for dressing wounds which in most cases would have been septic (Williams, 1982) starts to make sense. Much remains to be done, however, before the healing properties of *Sphagnum* moss tissue can be fully understood. Sphagnan is essentially a kind of pectin, and there is an extensive literature on the immunostimulatory properties of complex pectins from a wide variety of higher plants, including some that are used specifically for wound healing

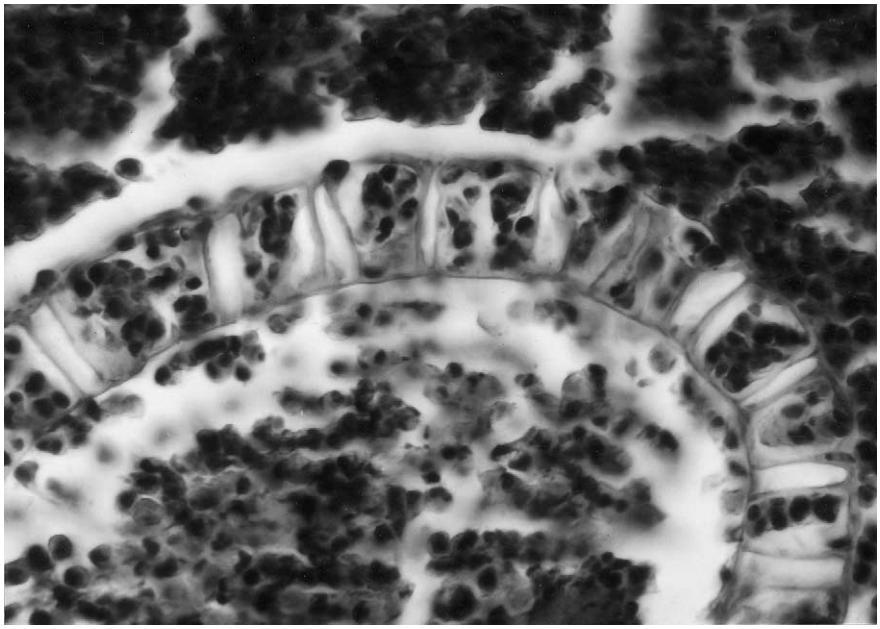

Fig. 2. Proliferation of healthy, viable leukocytes (blue-black) inside and around the hyaline cell walls of *Sphagnum palustre* (green) at the interface between a wound created surgically in a pig and a wad of *Sphagnum* held in place by a plastic ('Op-Site') occlusion for 3 days. There is no evidence of toxicity. Stain, Masson TriChrome; magnification, 800×; photo, Sheila E. Barnett.

in traditional African medicine (Paulsen, 2001; Diallo et al., 2001). Preliminary studies on the structure of sphagnan (Andresen et al., 1987) have revealed many similarities with these pectins.

4. Conclusions

1. *Sphagnum* wound dressings combat infection by immobilizing bacterial cells and depriving them of their nutrients.
2. Because of this mechanism there is no realistic possibility of pathogens' developing resistance to the antimicrobial action of sphagnan.
3. All pathogens, irrespective of identity, are subject to the action of sphagnan.
4. Sphagnan is not toxic to living cells and cannot therefore be expected to have any side-effects on the patient.
5. *Sphagnum* wound dressings must be changed often enough to ensure that an adequate surplus of active sphagnan is present at any time.

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